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Neurotrophin 3 rescues neuronal precursors from apoptosis and promotes neuronal differentiation in the embryonic metanephric kidney

(metanephros/kidney differentiation/neurotrophin receptor)

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ABSTRACT **We analyzed the developmental regulation and role of the neurotrophins during metanephric kidney morphogenesis. RNase protection assay revealed the presence of nerve growth factor, neurotrophin 3 (NT-3), and brain-derived neurotrophic factor mRNAs and the regulation of their expression during embryonic development of rat metanephros. NT-3 induced differentiation (neurite outgrowth) and survival (inhibition of apoptosis) of the neuronal precursors in cultured nephrogenic mesenchymes and neuronal differentiation in cultured whole kidneys, whereas NT-4/5, brain-derived neurotrophic factor, and nerve growth factor were without effect. The neurotrophins did not trigger tubular differentiation of isolated nephrogenic cells, which underwent apoptosis when cultured with or without the neurotrophins. NT-3 is thus an inducer of differentiation and a survival factor for renal neuronal cells, but none of the neurotrophins is a morphogen in kidney tubule induction.**

The neurotrophins trigger neurite outgrowth and maintain neuronal cells (for reviews, see refs. 1–3). They have been suggested to affect neuronal precursor cells by inducing proliferation of migratory neural crest cells and by triggering differentiation of neuroblasts (4–6). During embryonic development, different neurotrophins can act sequentially, for instance, on embryonic sensory neurons (6, 7) and motor neurons (8, 9). In nonneuronal cells, nerve growth factor (NGF) in combination with other factors influences the differentiation of bone marrow cells (10–12), and the action of the neurotrophins is, thus, not completely restricted to the nervous system. It has been suggested that they could act as signal substances in morphogenetic tissue interactions (13).

The family of neurotrophins consists of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT) 3, and NT-4/5. BDNF expression is restricted to certain areas of brain, lung, heart, and muscle (14); NGF is found in a variety of adult tissues including kidney; and practically all tissues express NT-3 (15, 16). The level of NT-3 mRNA in adult kidney is higher than it is in any other peripheral tissue (15). The NT-4/5 mRNA is expressed in rat kidney from embryonic day (E) 13 and is decreased by 4-fold in the adult kidney (17).

The neurotrophins bind to two classes of cell surface receptors (18, 19). The trkA, B, and C receptor tyrosine kinases serve as high-affinity receptors for the neurotrophins (20–23). trkC is the high-affinity receptor for NT-3 (24). All neurotrophins bind with a low affinity to a 75-kDa low-affinity NGF receptor (LNGFR) that does not contain a tyrosine kinase domain (25, 26) and is homologous to tumor necrosis factor receptors (27). The function of LNGFR is not fully resolved, but Rabizadeh *et al.* (28) have shown a putative role for LNGFR in the regulation of apoptosis of NGF-dependent nerve cells. Antisense oligonucleotide inhibition of LNGFR expression blocks kidney differentiation in organ cultures (29). In an attempt to evaluate the contribution of the neurotrophins to embryonic growth and differentiation, we have investigated the function and developmental regulation of NGF, BDNF, NT-3, and NT-4/5 in the embryonic rat metanephric kidney.

MATERIALS AND METHODS

mRNA Purification and cDNA Probes. RNA was prepared from ≈100 E13 to E14 rat kidneys, 40 E17 to E18 embryonic kidneys, and one adult kidney. Extraction and purification of mRNA were performed by using the Quick mRNA preparation kit (Pharmacia) by the manufacturer's protocol. cDNA probes were obtained by reverse transcription-coupled PCR as described (30). All PCR reagents were purchased from Cetus. The amplification program was as follows: 1 cycle at 94°C for 2 min, 55°C for 2 min, and 72°C for 4 min; 35 cycles for 1 min at 94°C, 1 min at 55°C, and 90 sec at 72°C; and the extension after the last cycle for 10 min at 72°C. PCR primers had the following sequences: NGF forward, 5′-TAGCGTAATGTCCATGTTGT, and reverse, 5′-CCCACACACTGACACTGTCA; BDNF forward, 5′-CGGCCCAATGAGGAAAACAATAAG, and reverse, 5′-CAGTTGGCCTTTCGAGACGGGGAC; NT-3 forward, 5′-GGCAACAACATGGATCAAAGGAGT, and reverse, 5′-CTGTTGCCGTAGTAGTTCTGTGTC; NT-4/5 forward, 5′-TGAGTGGCTGGGTGCACCGACC, and reverse, 5′-GAGAGTCTGCAGTCAACGCCG.

PCR-amplified fragments of rat NGF, NT-3, BDNF, NT-4/5, and actin cDNAs were blunt-end-cloned into the *Sma* I site of the pGEM4Z vector (Promega). After cloning, the specificity of all fragments was verified by direct sequencing. NT-3-specific complementary RNA was synthesized from a 187-bp cDNA fragment corresponding to the sequence encoding rat prepro-NT-3 mRNA at positions 145–331 (15). The rat BDNF cDNA fragment of 299 bp corresponds to the nt 320–619 of the rat BDNF cDNA (31). Rat NGF complementary RNA was synthesized from a 434-bp cDNA fragment corresponding to rat NGF mRNA nt 283–716 (32), and rat NT-4/5 cDNA was 256 bp long and corresponded to the sequence of rat NT-4/5 cDNA at positions 2195–450 (20).

RNase Protection Assay. ^{32}P-labeled antisense RNA fragments were synthesized by using the Riboprobe Gemini kit (Promega) and specific bands were gel-purified by polyacrylamide gel electrophoresis on 5% gels. RNA amounts from

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Abbreviations: NGF, nerve growth factor; NT, neurotrophin; BDNF, brain-derived neurotrophic factor; LNGFR, low-affinity NGF receptor; BrdU, bromodeoxyuridine; NF, neurofilament; E, embryonic day.

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different embryonic stages were equalized. RNase protection was done by using an RPA kit (Ambion, Austin, TX) by the protocol of the manufacturer. NGF and NT-3 probes had a linker sequence and were, therefore, longer than the BDNF probe. A 175-bp rat actin control was obtained by PCR of rat genomic DNA.

Tissue Culture, Growth Factors, and Immunocytochemistry. E13 embryonic rat metanephric kidneys were microsurgically separated, and the nephrogenic mesenchymes were enzymatically removed from the ureter bud as described (33). Whole kidneys and isolated kidney mesenchymes were cultured up to 6 days in Trowell-type cultures in Eagle's minimum essential medium (GIBCO) supplemented with 10% (vol/vol) fetal calf serum (Myoclone, GIBCO). NGF was from Sigma. Murine BDNF and NT-3 were from Yves-Alan Barde (Max-Planck-Institute for Psychiatry, Munich). Also, commercially available human NT-3 and NT-4/5 (PeproTech) were used. The neurotrophins were added to the culture medium at day 0 of culture and changed every second day. All neurotrophins were used at 25 ng/ml (8). The harvested tissue explants were fixed with ice-cold methanol for 10 min and stained with polyclonal antibody against L1 neural cell adhesion protein (34) from Melitta Schachner (Eidgenössiche Technische Hochschule, Zurich), monoclonal antibody to LNGFR (Boehringer Mannheim), monoclonal antibody 13AA8 to neurofilaments (35), polyclonal antibodies to trkC (Santa Cruz Biotechnology), and Hoechst fluorochrome 33342.

The number of the cells and their nuclear morphology were determined by Hoechst fluorochrome staining of the nuclei. Mesenchymes from six E13 rat kidneys were analyzed for each concentration of the neurotrophins at day 6. Bromodeoxyuridine (BrdU) incorporation and detection kit was used to analyze mesenchymes by the manufacturer's protocol (Amersham). The number of BrdU-labeled cells and the total number of cells were counted from the stained preparations, and the proliferation index is given in percentage as the ratio of the number of BrdU-positive cells/the total number of cells in the explant × 100. The cell cycle distribution of eight kidney mesenchymes in culture medium was also analyzed by Becton Dickinson fluorescence-activated cell sorter after ethidium bromide labeling of the nuclei (CellFIT Cell-Cycle Analysis version 2.01.2). To visualize apoptotic cells in cultured kidneys, the Apoptag labeling kit (Oncor) was used by the manufacturer's protocol.

RESULTS

Effects of NGF, BDNF, NT-3, and NT-4/5 on Isolated Kidney Mesenchymes. Whole kidneys differentiate *in vitro*, but microsurgically isolated uninduced nephrogenic mesenchymes without the ureter bud flatten and within 3 to 4 days die through apoptosis (33, 36, 37). A microdissected rat kidney mesenchyme contains, by flow cytometric analysis, ≈8000 cells that are homogeneous by their morphology but represent two types of precursor cells: neuroblasts and tubulogenic cells (38). NT-3 increased the total number of surviving cells in the kidney mesenchymes cultured for 6 days, but no signs for epithelial differentiation were seen. NGF, BDNF, and NT-4/5 were without effect on cell number and morphology (Fig. 1).

The proliferation index in the cultured mesenchymes, as measured by BrdU incorporation, did not show any statistically significant differences between the neurotrophin-containing medium and control cultures. It was low, on average only 16%. The range between individual mesenchymes varied between 9 and 25%. Neurotrophin supplementation did not affect these values. Eight mesenchymes from control cultures were pooled and compared to eight mesenchymes pooled from neurotrophin-containing medium by flow cytometry analysis at time points shown in Fig. 2. In accordance with manual counting, none of the neurotrophins increased the proportion of S-phase

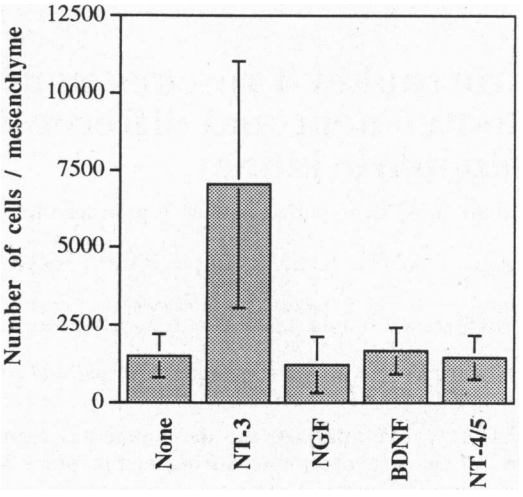

FIG. 1. Effect of the neurotrophins on the number of cells in a kidney mesenchyme. Six mesenchymes were counted from each medium containing the indicated addition, and the result is given as the mean number of cells in one mesenchyme at day 6 of culture. The bars represent the SEM.

cells. On the contrary, in day 1 cultures, fewer S-phase cells were seen with NT-3 than with control or other neurotrophins. As induction is always accompanied by the increase in the number of S-phase cells (39), thus, the data indicate that the neurotrophins had not induced epithelial differentiation.

To identify the neurotrophin-responsive cell type, we traced two marker proteins: L1 neural cell adhesion protein, expressed by the renal neuronal cells and the ureter bud, and neurofilaments (NFs), expressed by the renal neuronal and stromal cells (38). Coexpression of these proteins is only seen in the renal neuronal cells, which occur as single cells and in the renal microganglion in the E13 rat kidney (38). The number of neuronal precursor cells that do not express L1 and NFs could not be estimated. Nephrogenic mesenchymes were isolated from E13 kidneys. After 6 days in culture, isolated mesenchymes showed more cells positive for both L1 and NFs when cultured with NT-3 than with other neurotrophins or in control medium (Fig. 3). Double immunofluorescence staining showed also that the neurotrophin receptors trkC and LNGFR were expressed in a great majority of cells after 4 days of culture with NT-3 but not in control cultures (Fig. 4).

The occurrence of apoptosis in neuronal cells in cultured nephrogenic mesenchymes was investigated by Apoptag label-

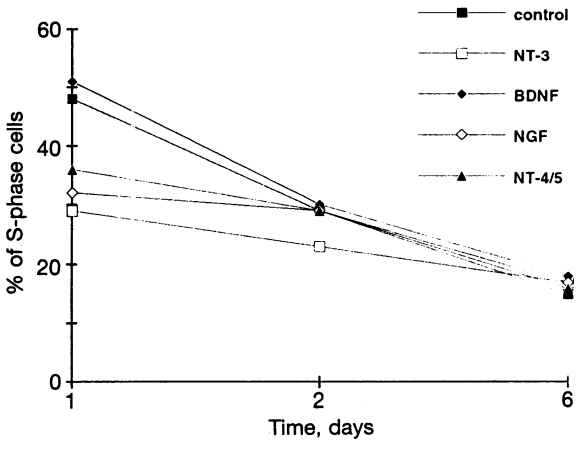

FIG. 2. Flow cytometry analysis of the percentage of S-phase cells in rat kidney mesenchymes cultured in the presence or absence of various neurotrophins as indicated.

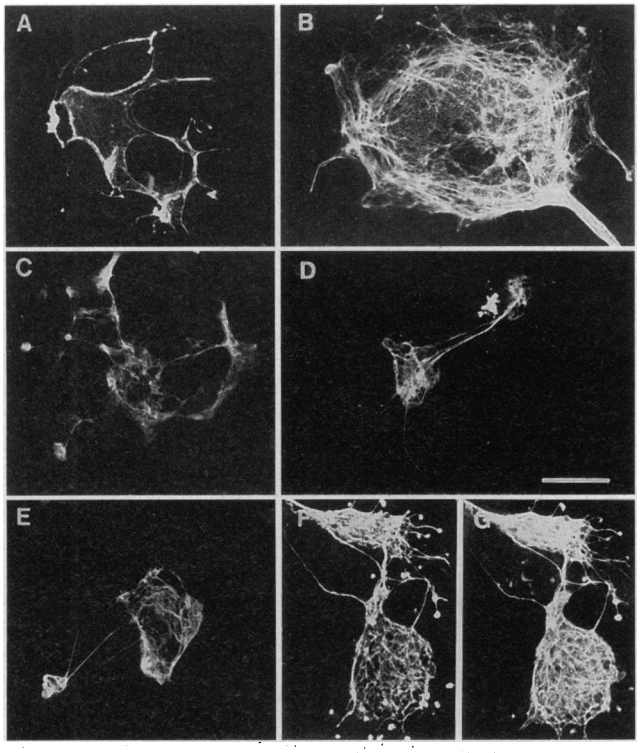

FIG. 3. Effect of the neurotrophins on L1 neural cell adhesion protein-positive cells in 6-day cultures of isolated kidney mesenchymes. (*A*) No neurotrophins. (*B*) NT-3. (*C*) NGF. (*D*) BDNF. (*E*) NT-4/5. (*F* and *G*) Double staining for NFs and L1, correspondingly, in the presence of NT-3. (Bar = 200 μm.)

ing. L1-positive cells in control cultures showed abundant apoptotic fragmentation and pycnosis of nuclei by day 3 (Fig. 5 *A, C,* and *E*), and similar results were obtained with NGF, BDNF, and NT-4/5 (data not shown). In contrast, the nuclear morphology of L1-positive cells in cultures in the presence of NT-3 was normal (Fig. 5 *B, D,* and *F*). NT-3 withdrawal activated regression of the NT-3-dependent neuronal cells during a further 6-day culture in normal medium (data not shown). Thus, NT-3 selectively rescued the neuronal precursor cells from apoptosis in the kidney mesenchymes and promoted their survival and neuronal differentiation. The L1-negative cells with low apoptosis label (seen in Fig. 5) may include some neuronal precursors, but mostly appear to be nonneuronal nontubulogenic cells of unknown character. This abundant apoptosis of L1-negative nephrogenic cells during the first days in culture (see ref. 38) prevented a detailed analysis of the apoptotic features in different cell types during this period.

The effect of the neurotrophins on kidney morphogenesis was further analyzed in cultures of whole E13 kidney rudiments that undergo epithelial differentiation *in vitro*. By day 3 of culture, NT-3 had expanded the size of the renal microganglion and enhanced neurite outgrowth, but such an effect was not seen without NT-3 or with other neurotrophins (data not shown). In contrast to the cultures with nephrogenic mesenchymes, no apoptosis labeling was seen in the neuronal cells of the whole kidney explants cultured with or without neurotrophins (data not shown), which indicated that the embryonic kidney may endogenously synthesize neurotrophins during epithelial differentiation of the nephrogenic mesenchyme and thereby become independent of exogenous neurotrophins.

RNase Protection Assay. The RNase protection assay was used to study the levels of neurotrophin mRNAs in embryonic and adult rat metanephros. Three developmental stages were studied, E13 to E14, E17 to E18, and adult kidneys. mRNAs for NGF, NT-3, and BDNF were detectable in early kidney

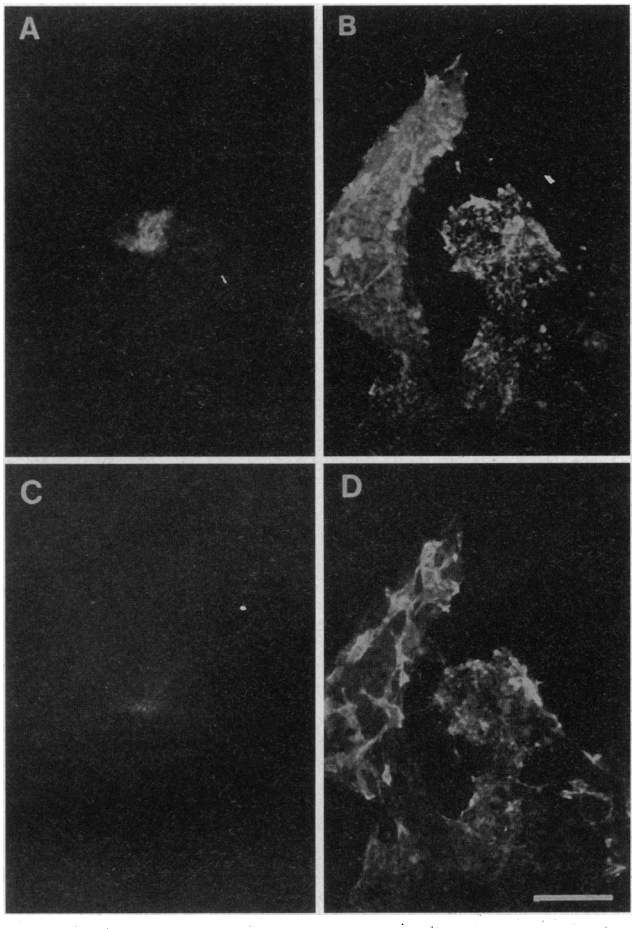

FIG. 4. Effect of NT-3 on trkC and LNGFR expression in cultured kidney mesenchymes. Double-immunofluorescence label with trkC (*A* and *B*) and LNGFR (*C* and *D*) antibodies. *A* and *C* depict a mesenchyme that was cultivated for 4 days without neurotrophins; *B* and *D* depict a mesenchyme cultivated with NT-3 at 25 ng/ml. (Bar = 100 μm.)

rudiments (Fig. 6); also NT-4/5 transcripts are expressed in the embryonic and adult rat kidneys (17). The levels of NGF and BDNF transcripts declined during kidney differentiation, the latter being undetectable in adult kidney. Conversely, the level of NT-3 mRNA slightly increased by age and was higher in adult kidney.

DISCUSSION

The development of the embryonic kidney is governed by sequential tissue interactions between the ureteric bud and the nephrogenic mesenchyme (39, 40). Heterologous recombination experiments have shown that the unknown signal molecules involved in kidney tubule induction are widely distributed among embryonic tissues, including the nervous tissue (32). Their molecular nature is not known but close cell-to-cell contacts are required for tubule induction (40). In organ culture, inhibition of LNGFR expression by antisense deoxyoligonucleotides perturbs kidney morphogenesis (29). Furthermore, the embryonic metanephric kidney rudiment contains neuronal precursor cells, their neuronal differentiation is temporally coupled with epithelial differentiation of the kidney, and the neuronal characteristics become apparent within the first days after kidney tubule induction (38). We show herein that the embryonic metanephric kidney expresses NGF, BDNF, and NT-3 mRNAs (for NT-4/5, see ref. 17). *In situ* hybridization (data not shown) demonstrated that NT-3 is expressed predominantly by the kidney tubules, and it is the

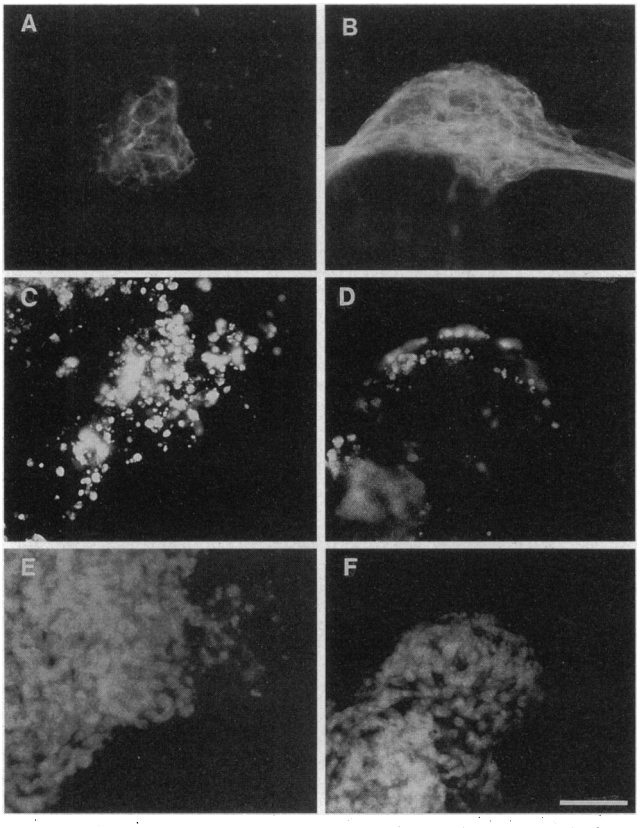

FIG. 5. Effect of NT-3 on apoptosis of neuronal cells in 3-day cultures of nephrogenic mesenchymes. (*Left*) Control culture without neurotrophins. (*Right*) Mesenchyme grown with NT-3. (*A* and *B*) L1 neural cell adhesion protein labeling of the neuronal cells. (*C* and *D*) Labeling of apoptotic nuclei by using the Apoptag staining kit. (*E* and *F*) Hoechst fluorochrome staining of all nuclei. By day 3 more L1-positive cells are seen in NT-3-treated than control mesenchymes, and only a few apoptotic cells are seen in the periphery of the neuronal cell cluster. (Bar = 40 μm.)

only neurotrophin found in abundance after terminal tubular differentiation. NGF is expressed by the smooth muscle cells of renal arteries. These completely different expression patterns suggest that NGF and NT-3 may have specific neuronal target cell populations within the embryonic kidney.

The relative number of neuronal cells in isolated nephrogenic mesenchymes was increased by NT-3, but it neither

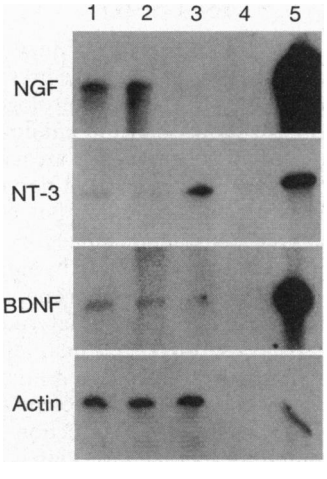

FIG. 6. RNase protection analysis of the neurotrophin mRNA levels at various stages of kidney differentiation. Lanes: 1, E13 to E14; 2, E17 to E18; 3, adult; 4, yeast RNA control; 5, undigested fragment.

affected the proliferation index during a 6-day culture nor induced epithelial differentiation of the nephrogenic mesenchymal cells. The proliferation index in the kidney mesenchyme is low before tubule induction and is increased only by the induction of epithelial differentiation (39). Because the neurotrophins did not induce tubulogenesis, it is not surprising that the proliferation rate remained low in the uninduced mesenchymes. However, the number of surviving neuronal cells, expressing both LNGFR and high-affinity neurotrophin receptor trkC, was increased by NT-3, which indicates that NT-3 primarily inhibits apoptosis in the neuronal precursors rather than induces their cell proliferation. No apoptosis was seen in the neuronal cells of the whole kidney cultures that undergo epithelial morphogenesis, suggesting that the neurotrophins synthesized by the differentiating embryonic kidney can rescue the renal neuronal precursor cells. In the whole kidney cultures, exogenous NT-3 enhanced neurite outgrowth and the size of the renal microganglion that is in accordance with the NT-3 responsiveness observed in the nephrogenic mesenchymes.

Only few L1- and NF-positive cells are seen in the metanephric kidney rudiment, and the number of neuronal precursor cells without L1 and NFs is not known at present. It is obviously less than the number of tubulogenic cells. A mitogenic response to a neurotrophin should be high in such a mixed cell population but we did not see it with the present techniques. Thus we cannot exclude that NT-3 might initially stimulate proliferation of the renal neuronal precursor cells, as NT-3 is known to stimulate proliferation of migrating neural crest cells (5). After migrating neuroblasts reach their destination, the local production of neurotrophins rapidly induces neuronal differentiation. In kidney mesenchyme cultures with NT-3, such a response became evident in 3 days. The few neuronal cells that survived and differentiated in kidney mesenchymes with other neurotrophins, or without neurotrophins, may be due to a low endogenous synthesis of neurotrophic factors in the uninduced nephrogenic mesenchyme. The present data clearly show that the neurotrophins are not morphogens in kidney tubule induction. However, their effect on morphogenesis after induction of the kidney mesenchyme cannot be excluded by present data. In this case their effects may be mediated via LNGFR rather than the high-affinity neurotrophin receptors, because only the former is expressed by the kidney tubules (29).

Regulation of LNGFR expression is poorly understood at present. NGF induces LNGFR expression in PC12 pheochromocytoma cells (26) and NT-3 in glial cells (41). In the embryonic kidney mesenchymes, NT-3 promotes the survival and differentiation of neuronal cells with LNGFR, but none of the neurotrophins induced LNGFR in the tubulogenic cells that express the receptor only after induction of differentiation. Thus, the LNGFR expression in the tubulogenic cells is independent of the action of the neurotrophins, unlike in PC12 and glial cells. Such a difference and the structure of the rat LNGFR promoter with testosterone- and retinoic acid-sensitive elements (42) indicate various regulatory systems for LNGFR gene expression.

The NT-3-dependent cells of the kidney mesenchyme express not only LNGFR but also trkC, the high-affinity receptor for NT-3. Our recent *in situ* hybridization data (data not shown) from embryonic kidneys show trkC transcripts in the peritubular stromal cells and microganglion in the hilus of the embryonic kidney, in addition to collecting tubules (43). Only microganglion neurons express L1 neural cell adhesion protein that characterizes the NT-3-responsive cells in the kidney mesenchyme (38). The renal cells responding to NT-3, therefore, represent the neuronal cells from the renal microganglion and mesenchyme rather than the stromal cells.

NT-3 is the predominant differentiation factor for neural crest cells in primary culture (5) and neural crest cells have

been traced to the embryonic metanephric kidneys by chicken–quail chimeras (44). As shown herein, NT-3 is the only neurotrophin that induces differentiation of the neuronal precursors in cultured kidney mesenchymes and rescues the renal neuronal cells from apoptosis. The present finding thereby further supports the proposal that the embryonic kidney rudiment contains cells of neural crest origin and the primary targets for NT-3 may be these neural-crest-derived cells.

In conclusion, NT-3 is the only neurotrophin that *in vitro* maintains the survival of the presumably neural-crest-derived neurons in the uninduced kidney mesenchyme and neuronal differentiation in the differentiating kidney.

BDNF and NT-3 were gifts from Dr. Yves-Alan Barde (Max-Planck-Institute for Psychiatry, Munich). Actin primers for PCR were from Dr. Olli Ritvos (University of Helsinki). Anti-L1 neural cell adhesion protein antibody was from Dr. Melitta Schachner (Eidgenössiche Technische Hochschule, Zürich). The 13AA8 antibody against NF proteins was from Dr. Ismo Virtanen (University of Helsinki). Ms. Satu Åkerberg and Ms. Birgitta Tjäder are acknowledged for their skilled technical assistance. This work was supported by grants from the Sigrid Jusélius Foundation, Academy of Finland, and the Finnish Cancer Societies.

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